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PROCEEDINGS OF THE NATIONAL ACADEMIE OF SCIENCES, USA, vol. 82, April 1985, pags 2468-2472; D.L. THIELE et al.: "Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes"

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FEDERATION PROCEEDINGS, vol. 44, no. 5, 8th March 1985, 69th annual meeting, Anaheim, California, 21th-26th April 1985, page 1688, abstract no. 7469; T. MEINEKE et al.: "Monocyte modulation of IL-2 induction of lymphokine activated killer cells"

CANCER, vol. 55, 1985, pages 1327-1333; A.A. RAYNER et al.: "Lymphokine-activated killer (LAK) cells"

NEW ENGLAND JOURNAL OF MEDICINE, vol. 313, no. 23, pages 1485-1492, 5th December 1985; S.A. ROSENBERG et al.: "Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer"

Description

FIELD

This invention pertains to adoptive immunotherapy, more particularly to the in vitro generation of human lymphokine activated killer cells for use in such therapy.

BACKGROUND

Adoptive immunotherapy has recently produced encouraging clinical results against some forms of cancer. See articles in the Wall Street Journal, April 9, 1987, and Time Magazine, April 20, 1987. The therapy involves removing peripheral blood from a patient, removing red blood cells (RBC's) from the blood to produce a lymphocyte-containing white blood cell (WBC) fraction, incubating the blood fraction in culture medium with interleukin-2 (IL-2) to produce activated, tumor-destroying lymphocytes called LAK cells, and injecting the LAK cells and additional IL-2 into the patient. In some cases IL-2 is injected into the patient before removal of the blood in order to stimulate production of lymphocytes.

One objection to adoptive immunotherapy is that it is very expensive. One reason it is expensive is that the current procedure for producing LAK cells is labor-intensive and time consuming. This procedure is described in Muul et al., "Large scale production of human lymphokine activated killer cells for use in adoptive immunotherapy, "Journal of Immunological Methods, 88:265-275 (1986). As described in Muul et al., in order to generate enough LAK cells for a single treatment about 2x10" lymphocytes were obtained by 10 successive leukaphereses of peripheral blood. In each leukapheresis, about 10-12 liters of whole blood were processed in an automated cell separator over a 4-hour period to produce a 400-500 ml leucocyte fraction. This fraction was diluted with 2 parts of a salt solution, then poured into 50 ml conical centrifuge tubes (40 ml/tube, approx. 30-40 tubes) and underlayed with 10 ml Ficoll-Hypaque solution. The contents were centrifuged, causing separation into a platelet-rich supernatant layer, a lymphocyte-rich layer, a Ficoll-Hypaque layer, a granulocyte layer and RBC layer. The supernatant was removed from each tube and discarded. The lymphocyte-rich fraction floating on the Ficoll-Hypaque was removed from each tube; these fractions were pooled and washed three times by suspension in salt solution and centrifugation. Since these steps must be repeated for each leukapheresis, 300-400 tubes must be handled for a single treatment.

Haemonetics Corporation of Braintree, Massachusetts, markets an automated blood cell separator known as the Haemonetics V-50, which utilizes a 2-port conically-shaped centrifuge bowl similar to the bowl described in U.S. Patent 3,145,713. The V-50 can be operated according to a standard leukapheresis protocol or according to a Surge® lymphocytopheresis protocol. The latter procedure, as described in U.S. Patents, 4,464,167 and 4,416,654, involves intermittent elutriation with previously-separated plasma, and is capable of providing more precise fractions of platelets, WBC's and RBC's than can be achieved with standard leukapheresis; it is referred to hereinafter elutriation leukapheresis.

For LAK cell processing, Haemonetics recommends use of the V-50 to separate a Buffy coat composed mostly of platelets and WBC's, followed by a secondary separation using Ficoll-Hypaque to provide a density gradient in the same centrifuge bowl for isolation of mononuclear cells(lymphocytes and monocytes) from the Buffy coat. Although this procedure is much less time-consuming and labor-intensive than the standard ficoll centrifugation described in Muul et al., it would be desirable to eliminate the ficoll separation step because it adds to the cost and can cause a reduced yield of lymphocytes. However, up to now it has been considered essential by those skilled in the art to conduct a ficoll separation in order to obtain a lymphocyte fraction sufficiently free of RBC's and granulocytes to be useful for production of LAK cells. It was assumed that RBC's and granulocytes would unduly interfere with the activation of the lymphocytes.

SUMMARY OF THE INVENTION

We have discovered that the step of ficoll density gradient centrifugation can be eliminated without unduly interfering with lymphocyte activation. Thus, our invention is an improvement in the method of producing LAK cells in vitro which comprises removing RBC's from whole blood to produce a lymphocyte-containing WBC-rich fraction and incubating the WBC-rich fraction in culture medium with IL-2 to activate the lymphocytes. The improvement comprises using the lymphocyte-containing WBC-rich fraction without intermediate separation of a lymphocyte and monocyte layer on a ficoll gradient.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the improved method for this invention, the RBC's can be removed in various ways. These include standard leukapheresis, elutriation leukapheresis, and centrifugation without use of ficoll. Ficoll is a synthetic water-soluble polysaccharide that has a weight average molecular weight of about 400,000 and that is widely used for the preparation of density gradients. It is available as such and in admixture with other substances under registered trademarks such as Ficoll-Paque, Ficoll-Hypaque and Ficoll-Isopaque. Leukapheresis refers to a process in which peripheral blood is withdrawn from a patient or donor, a WBC-rich fraction is separated out, and other blood fractions (plasma, platelets and RBC's) are returned to the source. Standard centrifugation is used to separate blood from donors into plasma, WBC-rich and RBC fractions which are stored for later use. (The term "Buffy coat" as used hereinafter refers specifically to the WBC-rich fraction obtained by standard centrifugation, although the term is also used in the art to refer to a platelet-rich, WBC-rich fraction obtained by leukapheresis.)

The various methods of removing RBC's produce WBC-rich fractions with varying amounts of residual RBC's and varying differentials. (The term "differential" or "diff" refers to the number percent of lymphocytes, monocytes and granulocytes based on the total number of those three cell types in a WBC-rich fraction.) Compositions of the various fractions will also vary depending upon the source. For example, a patient who has been primed with IL-2 may have a very high lymphocyte count. Typical ranges for the WBC-rich fractions obtained by various methods are compared with typical ranges for whole blood in the following table.

	No RBC	No WBC	Differential L	M	G
Standard Leukapheresis per 240 ml pack Vol. % RBC 10-20	10^{11} to $5x10^{11}$	$2x10^{9}$ to 10^{11}	60-80	5-25	5-25
Elutriation Leukapheresis per 400 ml pack Vol. % RBC 1-6	$2x10^{10}$ to 10^{11}	$2x10^{9}$ to 10^{11}	80-85	10-20	1-5
Buffy Coat per 40 ml pack Vol. % RBC 40-50	10^{11} to $3x10^{11}$	10^{8} to $2x10^{10}$	20-50	10-30	20-50
Normal Whole Blood per 450 ml unit	$1.6-2.4x10^{12}$	$2.3-4.6x10^{9}$	25-40	4-10	50-65

From the above table, it can be seen that lymphocyte-containing WBC-rich fractions usable in this invention can have RBC/WBC ratios from about 0.2 to about 300 and granulocyte contents from about 1% to about 50%. As a practical matter Buffy coats would principally be used for screening to determine whether a patient is capable of developing LAK cells. For generating LAK cells for use in adoptive immunotherapy the leukapheresis products having RBC vol. % of about 1-20% and RBC/WBC ratio of about 0.2-250 would be preferred.

At the present time, it is preferred to use the elutriation leukapheresis product because it is more nearly like the ficoll-separated products in both RBC and granulocyte content, and therefore would probably be more readily accepted by workers in the art. In addition, it appears that elutriation leukaphersis products can be cultured at a somewhat higher cell density (e.g., $1x10^{7}$/ml or higher) than can standard leukapheresis products on a routine basis. From the above table, it can be seen that elutriation leukaphersis products typically have a RBC/WBC ratio of about 0.2 to about 50, a RBC vol. percent of about 1-6 and a granulocyte content of 1-5. More typical ranges are RBC/WBC of about 0.5-25 and RBC vol. % of about 2-4.

Standard leukapheresis can be performed using instruments available from various manufacturers, including Haemonetics, Fenwall, and Cobe and following the manufacturers' instructions. The only instrument now available for performing elutriation leukapheresis is the Haemonetics V-50. Following the teaching of U.S. Patents 4,464,167 and 4,416,654 or the instructions provided by Haemonetics, the V-50 can be used to provide a WBC-rich fraction having low RBC and granulocyte content.

Monocyte content of the WBC-rich fraction can be reduced below the figures shown in the table by treatment of the leukapheresis product with an L-amino acid lower alkyl ester or hydrogen chloride salt thereof, e.g., methyl, ethyl, propyl, isopropyl, butyl, isobutyl, or t-butyl ester of phenylamaine, glutamic acid, glutamine or tyrosine. Phenyl alanine methyl ester is preferred. Further details are given in copending U.S. application Serial No. 868,697, filed May 30, 1986, and in the examples below.

Activation of the lymphocytes by incubation with IL-2 is accomplished in this invention in the same manner as in the prior art. Containers such as conventional flasks and roller bottles can be used, but the preferred containers are 0.2-5 liter tissue culture bags made from flexible copolymeric film materials as disclosed in copending application Serial No. 008,273, filed January 29, 1987. Most preferred is a bag made of a copolymer of 97 mol % ethylene and 1-octene. Any suitable culture medium can be used, but the preferred culture medium is RPMI 1640, which is described in "Culture of Animal Cells", Freshney, 72-73, Alan R. Liss, Inc., NY, supplemented with serum. Initial cell concentrations of up to about $1x10^7$ cells/ml can be used with an elutriation leukapheresis product and up to about $1x10^7$ cells/ml with a standard leukapheresis product. A concentration of at least $1x10^5$ cells/ml should be used for reason of economy. Preferred ranges would be $5x10^6$ to 10^7 cells/ml for elutriation leukapheresis products and $1\text{-}5x10^6$ cells/ml for standard leukapheresis products. The cells are incubated with IL-2 for about 2-7 days, preferably about 3-5 days, at a temperature of about 35-39 ° C, preferably 37 ° C.

"Interleukin-2" (IL-2) as used herein means human IL-2. It includes natural and recombinant IL-2 (rIL-2) and biologically functional equivalents thereof, such as the rIL-2 muteins disclosed in U.S. Patent 4,518,584. Preferably, the IL-2 is a rIL-2 composition consisting essentially of water, rIL-2 and, optionally, a polyol as described in assignee's copending application Serial No. 825,133, filed on January 31, 1986. Preferably, the IL-2 concentration in the culture medium is in the range of about $5x10^2$ to about $5x10^4$ pM, most preferably 1000 to 2000 pM.

The LAK cells prepared by the process of the invention can be suspended in a pharmaceutically acceptable carrier, such as saline, saline containing 5% normal human serum albumin, or Hank's balanced salt solution, to provide a composition which can be infused into a patient afflicted with a tumor. The patient is concurrently treated with rIL-2 as further described by Rosenburg et al., The New England Journal of Medicine 313, 1485-1492 (1985). In that modality, the patient's blood is withdrawn, subjected to leukapheresis and harvested cells are immediately cultured for 3 days to generate LAK cells. The LAK cells are then infused into the patient. Typically, about $3x10^{10}$ to $14x10^{10}$ LAK cells are infused in 4-9 doses. Interleukin-2 is administered every eight hours at doses such as 10,000, 30,000 or 100,000 units per kilogram of weight. The treatment consists of a two-week regime of leukapheresis and reinfusion and generally repetition starting the third week. Recombinant IL-2 can be included in the LAK cell composition.

Cytotoxicity (LAK) Assay

In the following examples, unless otherwise stated, a 4 hour ^{51}Cr release assay was used to measure cytotoxicity of LAK cells for tumor cells (LAK activity). Tumor cells at a concentration of about $2x10^6$ to $10x10^6$ per ml were incubated with 100 μCi of $Na_2\ ^{51}CrO_4$ in 0.4 mL of Tris-phosphate buffered saline for 1 hour at 37 ° C. The cells were washed 3 times with RPMI 1640 containing 5% or 10% fetal calf serum (FCS) and resuspended to 10^5 cells/mL in RMPI-20% FCS or RPMI-10% FCS. The effector cells (LAK cells) were suspended to various concentrations of 0.1 mL was added in to wells round bottom microliter plates. The ^{51}Cr labelled target cells (0.1 mL) were added to all wells. After 4 hours of incubation at 37 ° C, the plates were centrifuged and 0.1 mL of resulting supernatant was removed from each well and counted in a gamma counter. Percent cytolysis is calculated from the following formula:

$$\% \text{ cytolysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cmp}} \times 100$$

Each variable was tested in triplicate and the resulting data are expressed as % cytolysis. This cytotoxicity test is further described in "Selected Methods in Cellular Immunology," Mishell and Shiigi, eds., 124-137, W. H. Freeman and Co., San Francisco (1980).

In other experiments, the results of the assays are presented as "Lytic Units" (LU or LU30) which are the number of target cells per 100 effector cells when 30% of the target cells are killed when LAK cells and target cells are incubated together for 4 hours at 37 ° C. The calculation of LU is based upon the method of Pross et al., Journal of Immunological Methods 68, 235-249 (1984). The greater the number of LU, the

greater the potency of the LAK cell preparation.

All patents, patent applications and other printed publications cited in this application are incorporated herein by reference, especially the disclosure of U.S. Patents 4,464,167 and 4,416,654 relating to the production of a WBC-rich fraction by elutriation leukapheresis using previously separated plasma as elutriant.

EXAMPLE 1

Purpose:

1) To study the LAK activity of cells obtained from a Haemonetics V50 using the elutriation technique to obtain white blood cells.
2) To study the effects of phenyl alanine methyl ester (øAla) treatment and Ficoll treatment on the LAK activity of cells obtained from the Haemonetics V50 elutriation technique.

Cells:

Human lymphocytes (obtained from Haemonetics Corporation using V50 elutriation technique). Raji cells.

Materials:

1) Cell culture medium (CCM) = RPMI 1640 with 10% FBS, L-glutamine and Gentamicin
2) Phosphate buffered saline (PBS) 1x without Ca^{++} and Mg^{++}
3) Ficoll Hypaque (Ficoll)
4) øAla
5) Unopette® for WBC count
6) Ethylene butene copolymer Bag for cell culture
7) T25 tissue culture flasks
8) 1% NP 40
9) 2x TD buffer
10) ^{51}Cr (as sodium chromate)
11) recombinant Interleukin-2, 10 units/ml in 0.5M glucose (IL-2)
12) 96 well μ bottom tissue culture plate
13) SCS Harvesting System (Skatron)
14) Beckman Gamma 4000 Counter
15) Trypan Blue

Procedure:

A) Preparation of Cells

1) A total of 250 ml of a white blood cell fraction was collected from a Haemonetics V50 machine using the elutriation procedure as described in U.S. Patents 4,416,654 and 4,464,167.
2) A WBC count was performed using a Unopette®. The fraction contained $1.36x10^7$ WBC/ml and was estimated to contain approximately 3 vol. % RBC.
3) The cells were then brought to a concentration of $1x10^7$ WBC/ml (total volume = 340 ml).
4) 40 Ml of cells were put directly into culture (as described below).
5) The remaining 300 ml were treated with øAla (as described below).

B) øAla Treatment

1) Place 300 ml of cells into T150 flask.
2) Add 30 ml of øAla to cells
3) Mix well (gently).
4) Incubate at room temperature for 40 minutes.
5) After incubation, separate blood into 2 aliquots each containing 165 ml
   a) aliquot 1 was placed into culture

b) aliquot 2 was underlayed with Ficoll (as described below) and then placed into culture.

C) Set Up Culture

1) Cells Straight from V50 (No Ficoll; No øAla)

a) place 40 ml cells into a 50 ml centrifuge tube
b) centrifuge cells 10 minutes at 1200 rpm
c) discard supernatant
d) resuspend cells in CCM to a total volume of 40 ml
e) place desired amount of cells into T25 flasks
f) add CCM to flasks to bring white cells to desired concentration
g) add 5 µl IL-2 to each flask (final concentration 10 units/ml)
h) place flasks in 37° C incubator with 5% CO_2.

Set up 3 - T25 Flasks

$1x\ 10^6$ WBC/ml	$5X10^6$ WBC/ml	$1x10^7$ WBC/ml
1 ml cells	5 ml cells	10 ml cells
9 ml media (CCM)	5 ml media (CCM)	5 µl IL-2
5 µl IL-2	5 µl IL-2	

2) Aliquot 1 → Cells from V50 (øAla and No Ficoll)

a) place 165 ml of øAla treated cells into a 250 ml centrifuge tube
b) centrifuge for 10 minutes at 1200 rpm
c) discard supernatant
d) resuspend cells in 50 ml CCM
e) perform cell count using Unopette®; the WBC count was $1.9x10^7$ per ml
f) set up cultures in bags and flasks according to cell concentrations desired
g) place cultures in 37° C incubator with 5% CO_2.

Set Up 2 Cultures: T25 Flask $5x10^6$ cells/ml
Bag $9x10^6$ cells/ml

$$\frac{5x10^6}{1.9x10^7} = .260 \times 10 = \begin{cases} 2.6 \text{ ml cells} \\ 7.4 \text{ ml media (CCM)} \\ 5 \text{ µl IL-2} \end{cases} \text{ in T25 flask}$$

$$\frac{9x10^6}{1.9x10^7} = .474 \times 100 = \begin{cases} 47.4 \text{ ml cells} \\ 52.6 \text{ ml media (CCM)} \\ 50 \text{ µl IL-2} \end{cases} \text{ in Bag}$$

3) Aliquot 2 → Cells from V50(øAla and Ficoll)

a) place 40 ml of øAla treated cells into 4-50 ml centrifuge tubes
b) underlay blood with 10 ml of Ficoll

c) centrifuge for 30 minutes at 1900 rpm
d) collect interface layer with a sterile pasteur pipette and place cells into a sterile 50 ml centrifuge tube
e) bring volume in the tube up to 50 ml using PBS
f) centrifuge for 10 minutes at 1200 rpm
g) discard supernatant
h) resuspend pellet in 50 ml of CCM
i) centrifuge for 10 minutes at 1200 rpm
j) resuspend in 5 ml of CCM
k) perform cell count using trypan blue; then
l) set up cultures in bags and flasks according to cell concentrations desired
m) place cultures in 37° C incubator with 5% CO_2.

NOTE: No interface layer resulted after step 3; therefore, the cells were resuspended, re-underlayed with Ficoll and recentrifuged. After this, the cells in the interface were collected.

Set Up 3 Cultures: T25 Flask $5x10^6$ cells/ml
T25 Flask $10x10^6$ cells/ml
Bag $1.9x10^6$ cells/ml

$\frac{5x10^6}{6.8x10^7}$ = .074 x 10 = .740 ml cells
9.260 ml media (CCM) } in T25 flask
5 µl IL-2

$\frac{1x10^7}{6.8x10^7}$ = .147 x 10 = 1.47 ml cells
8.53 ml media (CCM) } in T25 flask
5 µl IL-2

$\frac{1.9x10^6}{6.8x10^7}$ = .0279 x 100 = 2.79 ml cells
97.21 ml media (CCM) } in T25 flask
50 µl IL-2

D) LAK Assay

The LAK assay was performed after cells were in culture for 4 days, according to the procedure given above.

NOTE: Due to the overabundance of red blood cells contained in the specimens, 3 specimens were treated with lysis buffer prior to the LAK assay.

Lysing solution: .83 g Ammonium Chloride
200 ml distilled H_2O

1) resuspended cell pellet in 10 ml lysing solution
2) incubate for 20 minutes at room temperature
3) centrifuge for 10 minutes at 1200 rpm
4) discard supernatant
5) resuspende in 1 ml of CCM
6) perform cell count
7) set up E:T ratios as described in LAK assay procedure.

DATA

Cell Counts

Specimen	Counts	Cells	Total Cells
Straight from V50	68	1.36×10^7	3.4×10^9/250 ml
After øAla	97	1.9×10^7	9.7×10^8/50 ml
After øAla and Ficoll	344	6.8×10^7	3.4×10^8/5 ml

Prepare Cells for øAla Treatment (Bring all cells to 1×10^7 cells/ml)

$$\frac{1 \times 10^7}{1.36 \times 10^7} = .735 \times 340 = 250 \text{ ml cells}$$

90 ml CCM

Take off 40 ml and put into culture.
Add 30 ml of øAla to remaining cells and incubate 40 minutes at room temperature.

Day 4 - ^{51}Cr Release Data

Specimen	Viable	Non-Viable	% Viable	Cells/mls	Mls of Cells	Mls of Media	Total Cells
Straight from V50							
*1x10^6 Flask	39	3	92%	7.8x10^6	.256	.744	
*5x10^6 Flask	113	44	87%	2.2x10^7	.091	.909	
F1x10^6 Flask	136			2.7x10^7	.074	.926	5.4x10^7
øAla No Ficoll							
*5x10^6 Flask	44	2	95%	8.8x10^6	.228	.772	
F9x10^6 Bag	92			1.8x10^7	.111	.889	3.6x10^7
øAla and Ficoll							
5x10^6 Flask	154	21	88%	3x10^7	.067	.933	
10x10^6 Flask	158	30	84%	3.2x10^7	.063	.937	6.4x10^7
1.9x10^6 Bag	48	2	92%	9.6x10^6	.208	.792	
Raji	82	5	94%	1.6x10^7	.240	39.760	

Straight From V50

1x10^6 Flask $\frac{2x10^6}{7.8x10^6} = .256$

5x10^6 Flask $\frac{2x10^6}{2.2x10^7} = .091$

10x10^6 Flask $\frac{2x10^6}{2.7x10^7} = .074$

øAla No Ficoll

$5x10^6$ Flask $\frac{2x10^6}{8.8x10^6} = .228$

$9x10^6$ Bag $\frac{2x10^6}{1.8x10^7} = .111$

øAla and Ficoll

$1x10^6$ Flask $\frac{2x10^6}{3x10^7} = .067$

$10x10^6$ Flask $\frac{2x10^6}{3.2x10^7} = .063$

$1.9x10^6$ Bag $\frac{2x10^6}{9.6x10^6} = .208$

Raji $= \frac{1x10^5}{1.6x10^7} = .006 \times 40 = .240$ ml cells
39.760 ml media

* specimens which were processed with lysing solution prior to LAK assay

F specimens counted using Unopette* method

Day 4 - ^{51}Cr Release Data

Maximum (Total) Release =	2011	Spontaneous Release =	463	21.0%
	2215		469	21.3%
	2361		573	26.0%
Avg =	2196 cpm	Avg =	502cpm	22.8%

Cells Straight From V50 - NoØAla and No Ficoll

Dilution	Flask $1x10^6$ CPM	Flask $1x10^6$ % Cytolysis	Flask $5x10^6$ CPM	Flask $5x10^6$ % Cytolysis	Flask $1x10^7$ CPM	Flask $1x10^7$ % Cytolysis
20:1	1011	30.1	1102	35.4	1222	42.5
	978	28.1	1143	37.9	1437	55.2
	1068	33.4	1142	37.8	1321	48.4
	mean	30.5	mean	37.0	mean	48.7
10:1	821	18.9	752	14.8	1014	30.2
	736	13.8	861	21.2	1072	33.7
	758	15.1	861	21.2	1361	50.7
	mean	15.9	mean	19.1	mean	38.2
5:1	613	6.6	757	15.1	907	23.9
	638	8.0	719	12.8	874	22.0
	591	5.3	747	14.5	815	18.5
	mean	6.6	mean	14.1	mean	21.5
2.5:1	512	0.6	635	7.9	584	4.9
	548	2.7	699	11.6	629	7.5
	495	-0.4	592	5.3	1012	30.1
	mean	1.0	mean	8.3	mean	14.2

ØAla and No Ficoll

Dilution	Flask $5x10^6$ CPM	Flask $5x10^6$ % Cytolysis	Bag $9x10^6$ CPM	Bag $9x10^6$ % Cytolysis
20:1	838	19.9	670	9.9
	716	12.7	962	27.2
	727	13.3	1158	38.7
	mean	15.3	mean	25.3
10:1	624	7.2	811	18.3
	550	2.9	723	13.1
	636	7.9	764	15.5
	mean	6.0	mean	15.6
5:1	541	2.3	719	12.8
	506	0.3	713	12.5
	654	9.0	822	18.9
	mean	3.9	mean	14.7

2.5:1	476	-1.5	709	12.2
	488	-0.8	713	12.5
	405	-5.7	727	13.3
	mean	-2.7	mean	12.7

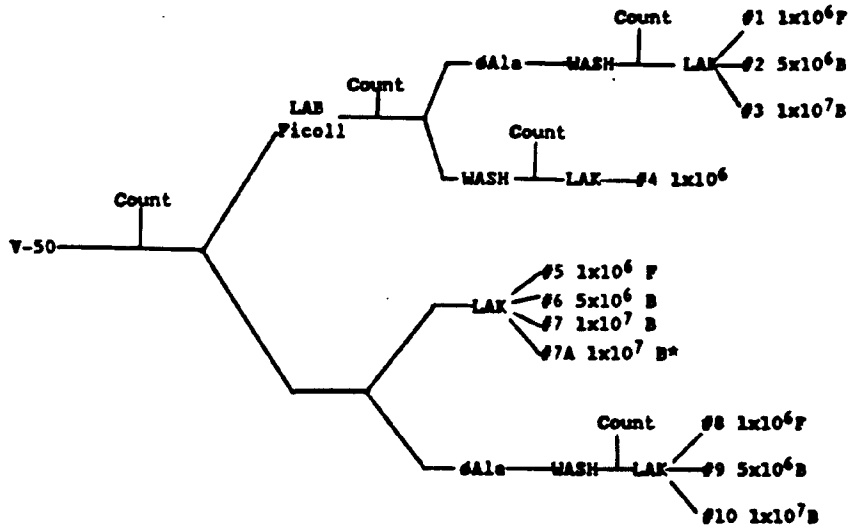
øAla and No Ficoll

	Flask $1x10^6$		Flask $5x10^6$		Flask $1x10^7$	
Dilution	CPM	% Cyto-lysis	CPM	% Cyto-lysis	CPM	% Cyto-lysis
20:1	1030	31.2	1239	43.5	1498	58.8
	1010	30.0	1200	41.2	1227	42.8
	1069	33.5	1288	46.4	1187	40.5
	mean	31.6	mean	43.7	mean	47.4
10:1	769	15.8	982	28.4	1125	36.8
	781	16.5	999	29.4	1188	40.5
	745	14.4	939	25.8	1261	44.8
	mean	15.5	mean	27.8	mean	40.7
5:1	623	7.2	876	22.1	1127	36.9
	624	7.2	755	15.0	953	26.6
	676	10.3	759	15.2	1326	48.7
	mean	8.2	mean	17.4	mean	37.4
2.5:1	422	-4.7	627	7.4	983	28.4
	464	-2.2	556	3.2	940	25.9
	426	-4.5	563	3.7	894	23.2
	mean	-3.8	mean	4.8	mean	25.8

EXAMPLE 2

Protocol:

The following diagram summarizes the protocol for this example.



F = T-25 Flask

B = Culture Bag

Procedure:

A) Separation of Cells

1) Cells were collected via elutriation technique on Haemonetics V-50.
2) A cell count was performed = $1.3x10^7$ cells/ml in 442 mls. $5.8x10^9$ total cells.
3) Cell Volume was split in two for processing.

B) LAB Ficoll

1) 221 mls of cells were mixed with PBS and layered on Ficoll.
2) Centrifuged 30 min. at 2000 rpm
3) Cells were then washed and counted
256 cells viable
99% Viability
1 nonviable cell
$5.1x10^8$ cells ml
$2x10^9$/40 ml
4) Set up cells in Culture for LAK @ $1x10^6$

Sample Code #	Conc. Wanted	Calculation	ml cells + ml Media + µl IL-2
#4	$1x10^6$	$1x10^6$/ $5.1x10^7x10$	= 0.2 ml + 9.8 ml + 5µl

5) The remaining of these Ficoll layered cells were set up for øAla
a) $V_1C_1 = V_2C_2$
(40 mls)($5.1x10^7$) = V_2 ($1x10^7$)
#mls total = V_2 = 200 mls
#mls media = V_2-V_1 = 160

#mls øAla = $V_2/9$ = 200/9 = 22.2 mlsøAla
b) Incubate 40 min and then wash.
c) Perform cell count and put cells in culture for LAK
Viable cells = 229
Nonviable = 6
% Viability = 97%
Cells/ml = 4.6×10^7
d) Set up cells for culture

Sample Code #	Conc. Wanted	Mls Cells + Ml media + µl IL-2
#1	$1x10^6$	0.20 ml + 9.8 mls + 5 µl
#2	$5x10^6$	10.9 mls + 89.1 ml + 50 µl
#3	$1x10^7$	21.8 ml + 78.2 ml + 50 µl

C) LAK directly from V-50

1) The second half of cells were used at this time. The amount of cells necessary to have cultures at a concentration of $1x10^6$, $5x10^6$ and $1x10^7$ were used and the remaining cells were diluted and treated with phenyl alanine methyl ester.

Sample Code #	Conc. Wanted	ml Cells + ml media + µl IL-2
#5	$1x10^6$	0.76 ml + 9.2 mls + 5 µl
#6	$5x10^6$	3.9 ml + 6.1 ml + 50 µl
#7	$1x10^7$	7.7 ml + 2.3 ml + 50 µl
*#7A	$1x10^7$	7.7 ml + 2.3 ml + 50 µl added

*10 ml sample - centrifuged - removed 5 mls plasma; added 5 mls media. Diluted cells to $1x10^7$.

D) øAla without Ficoll

øAla cells without separating with Ficoll first
$C_1V_2 = C_2V_2$
(200 ml)$(1.2x10^7) = (k)(1x10^7) = x = 260$
mls media = 60 mls
mls øAla = 29 mls.
Incubate 40 min.
Wash.
Perform cell count and put up in culture
Viable 240
%Viable 93%
Cells/ml = $4.8x10^7$
Total = $1.9x10^9$/40 ml

Sample Code #	Conc. Wanted	ml Cells + ml media + µl IL-2
#8	$1x10^6$	0.2 ml + 9.8 mls + 5 µl
#9	$5x10^6$	10.4 ml + 89.6 ml + 50 µl
#10	$1x10^7$	20.8 ml + 72.2 ml + 50 µl

All cultures were incubated for 3 days at 37°C and 5% CO_2. LAK - ^{51}Cr release performed. In the tables which follow, % REL means % Release, which is the same as % Cytolysis, calculated as explained above. E:T means the ratio of effector (LAK) cells to target (tumor) cells.

Cell counts were performed on all cultures.

Sample	Viable	Non-Viable	% Viable	Total
A	33	4	88	$6.5x10^6$/ml
B	99	8	93	$2.0x10^8$/10 ml
C	236	17	93	$4.7x10^8$/10 ml
D	7	0	100	$1.4x10^6$/ml
*E	22	1	96	$4.4x10^6$/ml
F	45	5	91	$1.8x10^7$/2 ml
G	38	4	90	$7.6x10^6$/ml
H	75	6	93	$1.5x10^8$/10 ml
J	141	15	90	$2.8x10^8$/10 ml
K	31	4	89	$6.2x10^6$/ml
# 1	34	1	97	$6.8x10^6$/ml
2	162	8	95	$3.2x10^8$10 /ml
3	375	20	94	$7.5x10^8$/10 ml
4	41	2	95	$8.1x10^6$/ml
5	27	2	93	$5.4x10^6$/ml
6	43	1	98	$1.7x10^7$/2 ml
7	121	17	88	$4.8x10^7$/2 ml
7A	149	9	94	$6.0x10^7$/2ml
8	50	0	100	$1x10^7$/ml
9	113	5	96	$2.3x10^8$/10 ml
10	424	47	90	$8.5x10^8$/10 ml

*Letter E had a heavy fibrin clot after centrifugation.

Raji tumor cells were prepared as target - Viability was 100% with a concentration of $3.6x10^5$/ml

Lab Ficoll - øAla

#1 @ $1x10^6$

E:T	CPM	% REL
20	500	59.06
20	501	59.23
20	470	54.03
10	377	38.42
10	372	37.58
10	326	29.87
5	233	14.26
5	288	23.49
5	253	17.62
2.5	197	8.22
2.5	198	8.39
2.5	196	8.05

#2 @ $5x10^6$

E:T	CPM	% REL
20	607	77.01
20	478	55.37
20	480	55.70
10	471	54.19
10	527	63.59
10	512	61.07
5	383	39.43
5	317	28.36
5	393	41.11
2.5	338	31.88
2.5	271	20.64
2.5	242	15.77

#3 @ $1x10^7$

E:T	CPM	% REL
20	603	76.34
20	647	83.72
20	571	70.97
10	546	66.78
10	408	43.62
10	405	43.12
5	351	34.06
5	333	31.04
5	324	29.53
2.5	265	19.63
2.5	217	11.58
2.5	271	20.64

STD Ficoll - No øAla

#4 @ $1x10^6$

E:T	CPM	% REL
20	565	69.97
20	507	60.23
20	529	62.42
10	483	56.21
10	400	42.28
10	462	52.68
5	276	21.48
5	255	17.95
5	291	23.99
2.5	288	23.49
2.5	263	19.30
2.5	235	14.60

Elutriation - No Ficoll or øAla

#5 @ $1x10^6$

E:T	CPM	% REL
20	588	73.83
20	496	58.39
20	466	53.36
10	305	26.34
10	296	24.83
10	339	32.05
5	285	22.99
5	281	22.32
5	228	13.42
2.5	211	10.57
2.5	229	13.59
2.5	237	14.93

#6 @ $5x10^6$

E:T	CPM	% REL
20	759	102.52
20	626	80.20
20	512	61.07
10	543	66.28
10	533	64.60
10	521	62.58
5	373	37.75
5	474	54.70
5	408	43.62
2.5	224	12.75
2.5	341	32.38
2.5	266	19.80

#7 @ $1x10^7$

E:T	CPM	% REL
20	314	27.85
20	288	23.49
20	312	27.52
10	203	9.23
10	186	6.38
10	206	9.73
5	187	6.54
5	185	6.21
5	177	4.87
2.5	157	1.51
2.5	190	7.05
2.5	167	3.19

#7A @ $1x10^7$

E:T	CPM	% REL
20	481	55.87
20	308	26.85
20	297	25.00
10	249	16.95
10	281	22.32
10	252	17.45
5	197	8.22
5	144	-0.67
5	167	3.19
2.5	160	2.01
2.5	197	8.22
2.5	164	2.68

Elutriation - øAla, No Ficoll

#8 @ $1x10^6$

E:T	CPM	% REL
20	592	74.50
20	574	71.48
20	463	52.85
10	377	38.42
10	438	48.66
10	425	46.48
5	257	18.29
5	228	13.42
5	431	47.48
2.5	207	9.90
2.5	217	11.58
2.5	211	10.57

#9 @ $5x10^6$

E:T	CPM	% REL
20	687	90.44
20	573	71.31
20	598	75.50
10	636	81.88
10	609	77.35
10	647	83.72
5	255	17.95
5	284	22.82
5	352	34.23
2.5	264	19.46
2.5	346	33.22
2.5	315	28.02

#10 @ $1x10^7$

E:T	CPM	% REL
20	572	71.14
20	586	73.49
20	493	57.89
10	349	33.72
10	288	23.49
10	254	17.79
5	183	5.87
5	204	9.40
5	233	14.26
2.5	205	9.56
2.5	214	11.07
2.5	195	7.89

EXAMPLE 3

A standard leukapheresis product containing 225 mls human leukocytes prepared from 3600 mls whole blood collected in 550 mls anticoagulant ACD-B was obtained from Biological Speciality Corporation, Lansdale, PA. The following procedures were performed using this product.

1) Set up a Unopette® (WBC) and a differential.
Differential:
90% Lymphocytes
6% Monocytes
4% Granulocytes
Direct = 165 cells
$3.3x10^7$ cells/ml
$7.4x10^9$ cells/225 mls
2) Set up cells in culture for LAK
@ $1x10^6$ = 0.30 ml cells + 9.70 ml media +5 µl IL-2
@ $5x10^6$ = 1.52 ml cells + 8.48 ml media +5 µl IL-2
@ $1x10^7$ = 3.04 ml cells + 6.96 ml media + 5 µl IL-2
Incubated @ 37° C, 5% CO_2 for 4 days.
3) Next 20 mls of cells were removed from the remaining cells and mixed with 20 mls of PBS without Ca^{++} and Mg^{++}. 40 mls of this mixture was layered onto 40 mls of Ficoll and centrifuged for 1/2 hour. Removed mononuclear cell layer and washed these cells 3 times. Performed 90 min. monocyte adherence. Washed 2 more times and performed cell count and put in culture for LAK. This is the Standard Sample.
Standard Cell Count:
Viable = 155
Non-viable = 1
% Viable = 99%
Cells/ml = $3.1x10^7$/ml
Total = $7.7x10^8$/25 ml.
Dilution for cell conc. of $1x10^5$ = 0.32 ml cells + 9.68 ml media + 5 µl (IL-2).
4) The remaining cells (200 mls) were then diluted with 460 mls CCM to bring the cell count to $1x10^7$/ml, and treated with 73 mls øAla.
Incubated at R.T. for 40 min.
Cells clotted during incubation.

Removed as much unclotted suspension as possible

- - washed and counted cells with WBC Unopette®.
  26 cells viable
  1 cell nonviable
  96% viability
  $5.2x10^6$ cells/ml
  $1.04x10^9$ cells/200 mls.

Put cells up in a bag at $5x10^6$ = 48 mls cells + 2 ml media + 25 µl IL-2.
Incubated at 37° C 5% CO_2 for 4 days for LAK.

5) Cell Counts After Incubation

	Viable	Non-Viable	% Viable	Cells/ml	Total
Std.	25	1	96	$4.9x10^6$	---
Direct @ $1x10^6$	27	1	96	$5.3x10^6$	---
Direct @ $5x10^6$	11	1	92	$2.1x10^6$	$1.1x10^7$/5ml
Direct @ $1x10^7$	24	5	83	$4.8x10^6$	$4.8x10^7$/10ml
øAla @ $5x10^6$	19	0	100	$3.8x10^6$	
Raji	85	7	92	$1.7x10^7$	

E:T ratio 20:1, 10:1, 5:1, 2.5:1

Dilutions

$2x10^6$

Std.	0.41 mls cells + 0.59 mls media
Direct $1x10^6$	0.38 ml cells + 0.62 ml media
Direct $5x10^6$	0.95 ml cells + 0.05 ml media
Direct $11x10^7$	0.42 ml cells +0.58 ml media
øAla	0.53 ml cells + 0.47 ml media
Raji	0.12 ml cells + 19.88 ml media

Results:

Total and Spontaneous CPM

E:T	Pos.	Code	CPM	% Cytolysis
Blank	1	BLAN	-5.9	.0
	2	BLAN	-8.6	.0
	3	BLAN	-10.3	.0
	0	MEAN	-8.3	.0
Max.	4	TOTA	823.0	.0
Release	5	TOTA	700.9	.0
	6	TOTA	896.9	.0
	0	MEAN	806.9	100.0
Spont.	7	REFR	104.5	13.0
Release	8	REFR	97.5	12.1
	9	REFR	109.3	13.5
	0	MEAN	103.8	.0

Standard Ficoll 1x10^6

20:1	10 UNKS	276.5	24.6
	11 UNKS	287.2	26.1
	12 UNKS	309.1	29.2
	0 MEAN	291.0	26.6
10:1	13	226.2	17.4
	14	212.4	15.4
	15	255.8	21.6
	0 MEAN	231.4	18.2
5:1	16	174.4	10.0
	17	180.4	10.9
	18	162.4	8.3
	0 MEAN	172.4	9.8
2.5:1	19	134.2	4.3
	20	124.8	3.0
	21	99.6	-.6
	0 MEAN	119.5	2.2

Direct - No Ficoll 1x10^6

20:1	22	490.6	55.0
	23	436.5	47.3
	24	423.7	45.5
	0 MEAN	450.3	49.3
10:1	25	484.5	54.1
	26	526.7	60.1
	27	495.8	55.8
	0 MEAN	502.4	56.7
5:1	28	391.6	40.9
	29	395.3	41.5
	30	432.2	46.7
	0 MEAN	406.4	43.0
2.5:1	31	236.5	18.9
	32	298.4	27.7
	33	220.7	16.6
	0 MEAN	251.9	21.1

Direct - No Ficoll 5x10^6

20:1	34	409.2	43.4
	35	370.9	38.0
	36	361.2	36.6
	0 MEAN	380.5	39.3

10:1	37		352.4	35.4
	38		409.3	43.4
	39		364.4	37.1
	0	MEAN	375.3	38.6
5:1	40		313.3	29.8
	41		293.3	27.0
	42		292.5	24.0
	0	MEAN	293.1	26.9
2.5:1	43		194.5	12.9
	44		227.2	17.6
	45		194.9	13.0
	0	MEAN	205.5	14.5

Direct - No Ficoll $1x10^7$

20:1	46		245.6	20.2
	47		193.2	12.7
	48		195.7	13.1
	0	MEAN	211.5	15.3
10:1	49		191.3	12.4
	50		188.6	12.1
	51		232.2	18.3
	0	MEAN	204.1	14.3
5:1	52		174.1	10.0
	53		166.8	9.0
	54		161.8	8.2
	0	MEAN	167.6	9.1
2.5:1	55		136.0	4.6
	56		149.8	6.5
	57		114.9	1.6
	0	MEAN	133.6	4.2

øAla $5x10^6$

20:1	58		278.1	24.8
	59		221.1	16.7
	60		226.6	17.5
	0	MEAN	241.9	19.6
10:1	61		206.5	14.6
	62		188.9	12.1
	63		195.9	13.1
	0	MEAN	197.1	13.3
5:1	64		186.5	11.8
	65		199.8	13.7
	66		173.3	9.9
	0	MEAN	186.5	11.8

2.5:1	67	199.2	13.6
	68	175.1	10.1
	69	212.7	15.5
	0 MEAN	195.7	13.1

EXAMPLE 4

A standard leukapheresis product containing 230 mls human leukocytes prepared from 3600 ml whole blood collected in 520 mls anticoagulant ACD-B was obtained from Biological Specialty Corporation, Lansdale, PA. The following procedures were performed using this product.

1) Removed 10 mls of cells

A)
1) Took 5 mls of this blood and mixed with 5 mls of PBS
2) Underlayered 10 mls of Ficoll
3) Centrifuged for 30 min @ 2000 rpm
4) Washed and counted
5) This was the Standard @ $1.5x10^6$ cells/ml in 10 ml flask

Standard
Viable = 49
Non-viable = 0
% Viable = 100%
Cells/ml = $9.8x10^5$/ml
Total = $1.96x10^8$/20 ml.
Dilution: 1.5 ml cells + 8.5 mls media + 1 µl IL-2

B)
1) The second 5 mls was used for direct testing
2) A WBC (via Unopette®) and differential were performed:
3) WBC $4.5x10^7$/ml
$2.25x10^8$/5 ml
Diff. 72% Lymphocytes
22% Granulocytes
6% Monocytes
4) Cells were then put up in culture at $1.5x10^6$/ml and $5x10^6$/ml in 10 ml flasks
$1.5x10^6$/ml = 0.33 ml/cells + 9.67 ml media + 1 µl IL-2
$5x10^6$/ml = 1.11 ml/cells + 8.89 ml media + 1 µl IL-2
Cells were incubated 4 days; chromium release assay was run.

Cell Count:

	Viable	Non-Viable	% Viable	Cells/ml
Standard $1.5x10^6$	40	3	93	$8x10^6$
Direct @ $1.5x10^6$	40	2	94	$8x10^6$
Direct @ $5x10^6$	24	1	96	$4.8x10^6$
Raji	40	6	87	$8x10^6$

3) LAK Assay

E:T ratio 40:1, 20:1, 10:1, 5:1, 2.5:1 1.25:1
Cells were diluted to $4x10^6$

Raji's were diluted to $1x10^5$

Dilutions	
Std.	0.5 ml cells + 0.5 ml media
Direct $1.5x10^6$	0.5 ml cells + 0.5 ml media
Direct $5x10^6$	0.83 ml cells + 0.17 ml media
Raji	0.25 ml cells + 19.75 ml media

Results:

Total and Spontaneous CPM

E:T	Pos. Code	CPM	% Cytolysis
Blank	1 BLAN	-11.0	.0
	2 BLAN	-10.6	.0
	3 BLAN	-10.6	.0
	0 MEAN	-10.7	.0
Max. Release	4 TOTA	576.9	.0
	5 TOTA	564.6	.0
	6 TOTA	584.6	.0
	0 MEAN	575.4	100.0
Spont. Release	7 REFR	139.7	24.3
	8 REFR	140.6	24.4
	9 REFR	152.3	26.5
	0 MEAN	144.2	.0
	Standard $1.5x10^6$		
40:1	10 UNKS	540.1	91.8
	11 UNKS	547.0	93.4
	12 UNKS	518.3	86.8
	0 MEAN	535.2	90.7
20:1	13	459.5	73.1
	14	503.2	83.3
	15	458.0	72.8
	0 MEAN	473.6	76.4
10:1	16	404.5	60.4
	17	410.8	61.8
	18	439.1	68.4
	0 MEAN	418.1	63.5
5:1	19	320.5	40.9
	20	279.1	31.3
	21	275.0	30.3
	0 MEAN	291.5	34.2
2.5:1	22	222.1	18.1
	23	253.0	25.2
	24	232.1	20.4
	0 MEAN	235.7	21.2
1.25:1	25	175.7	7.3
	26	205.6	14.2
	27	207.9	14.8
	0 MEAN	196.4	12.1

Direct - No Ficoll $1.5x10^6$

80:1	28		415.5	62.9
	29		393.0	57.7
	30		461.8	73.7
	0	MEAN	423.4	64.8
40:1	31		417.7	63.4
	32		404.2	60.3
	33		405.6	60.6
	0	MEAN	409.2	61.5
20:1	34		381.1	55.0
	35		407.0	60.9
	36		447.9	70.4
	0	MEAN	412.0	62.1
10:1	37		430.5	66.4
	38		407.4	61.0
	39		442.6	69.2
	0	MEAN	426.8	65.5
5:1	40		337.5	44.8
	41		357.4	49.5
	42		358.0	49.6
	0	MEAN	351.0	48.0
2.5:1	43		240.8	22.4
	44		270.9	29.4
	45		262.1	27.3
	0	MEAN	257.9	26.4
1.25:1	46		186.9	9.9
	47		194.1	11.6
	48		182.7	8.9
	0	MEAN	187.9	10.1

Direct - No Ficoll $5x10^6$

40:1	49		391.0	57.2
	50		392.1	57.5
	51		399.6	59.2
	0	MEAN	394.2	58.0
20:1	52		386.3	56.2
	53		381.1	54.9
	54		377.0	54.0
	0	MEAN	381.5	55.0

10:1	55		346.0	46.8
	56		346.0	46.8
	57		325.5	42.0
	0	MEAN	339.2	45.2
5:1	58		296.5	35.3
	59		236.6	21.4
	60		239.0	22.0
	0	MEAN	257.4	26.2
2.5:1	61		190.6	10.8
	62		172.8	6.6
	63		190.5	10.7
	0	MEAN	184.6	9.4
1.25:1	64		151.7	1.7
	65		172.5	6.6
	66		203.8	13.8
	0	MEAN	176.0	7.4

EXAMPLE 5

Materials:

Buffy coat - 52 mls of blood
Cell count - $4.3x10^7$ cells/ml (total WBC)
$1.8x10^7$ neutrophils/ml (42%)
(est.) $1\text{-}2x10^7$ lymphocytes/ml (20-50%)
(est.) $5x10^9$ RBC/ml
(est.) $0.5\text{-}1x10^7$ monocytes/ml
Ficoll-Hypaque (Ficoll)
CCM - 5% FCS - RPMI

Procedures:

1) No Ficoll

a) To 10.5 ml of Buffy coat add 215 ml CCM. Cell Count $2x10^6$ cells/ml (total WBC)
b) Add 10 µl/ml of IL-2
c) Place 112 ml of culture mix in flask
d) Place 112 ml of culture mix in bag
e) Incubate at 37° C for 20 days.
f) Sample at 3, 6, 12, 17 and 20 days for cell count and ^{51}Cr Release (LAK) assay.

2) Ficoll

a) Put 42 mls Buffy Coat in 50 ml centrifuge tube
b) Centrifuge at 800 g for 10 minutes
c) Discard supernatant, recover mononuclear WBC layer (Lymphocytes and monocytes) floating on Ficoll layer, wash 3X. $300x10^6$ total mononuclear cells isolated.
d) Add CCM to provide mononuclear cell concentration of $2x10^6$/ml
e) Place 75 ml in flask
f) Place 75 ml in bag
g) Incubate at 37° C for 20 days
h) Sample at 3, 6, 12, 17 and 20 days for cell count and ^{51}Cr release (LAK) assay.

Summary of Cell Counts (#x10^6/ml)

Days Culture	Flasks		Bags	
	Ficoll	No Ficoll	Ficoll	No Ficoll
0	2x10^6	2x10^6	2x10^6	2x10^6
3	1.5x10^6	0.9x10^6	2.1x10^6	.7x10^6
6	2x10^6	0.4x10^6	2x10^6	.8x10^6
12	2.5x10^6	1.4x10^6	2.7x10^6	2x10^6
17	1.6x10^6	1.2x10^6	1.9x10^6	1.1x10^6
20	1.1x10^6	0.6x10^6	2.4x10^6	1.2x10^6

Summary of LAK Activity

$^3LU_{30}$

Days Culture	Flasks		Bags	
	Ficoll	No Ficoll	Ficoll	No Ficoll
3	10	40	5	20
6	5	100	5	14
12	2.5	1	2	<1
17	7	<1	7	1
20	7	2	2.5	1

Claims

1. In the method of producing LAK cells in vitro which comprises removing RBC's and plasma from whole blood to reduce a lymphocyte-containing WBC-rich fraction and incubating the WBC-rich fraction in culture medium with IL-2, the improvement which comprises removing RBC's and plasma and using the WBC-rich fraction without an intermediate separation of lymphocytes on a ficoll gradient.

2. Method of Claim 1 wherein the RBC's are removed by leukapheresis and the volume percent of RBC's in the WBC-rich fraction is in the range of about 1-20.

3. Method of Claim 2 wherein the RBC/WBC ratio in the WBC-rich fraction is in the range of about 0.2-250.

4. Method of Claim 1 wherein the RBC's are removed by elutriation leukapheresis and the volume percent of RBC's in the WBC-rich fraction is in the range of about 1-6.

5. Method of Claim 4 wherein the RBC/WBC ratio in the WBC-rich fraction is in the range of about 0.2-50.

6. Method of Claim 5 wherein the WBC differential is about 80-85% lymphocytes, about 10-20% monocytes, and about 1-5% granulocytes.

7. Method of Claim 6 wherein the volume percent RBC's in the WBC-rich fraction is in the range of about 2-4, the RBC/WBC ratio in the WBC-rich fraction is in the range of about 0.5-25.

8. Method of Claim 1 wherein the monocytes are depleted by treatment with phenyl alanine methyl ester

before incubation of the WBC-rich fraction.

9. Method of Claim 2 wherein the WBC-rich fraction is washed with salt solution prior to incubation to inhibit clotting.

10. In the method of generating LAK cells by incubating a lymphocyte-containing WBC-rich fraction in culture medium with IL-2, the improvement which comprises using a lymphocyte-containing WBC-rich fraction having a RBC/WBC ratio by number in the range of about 0.2 to 300 and RBC volume percent of about 1-50.

11. Method of Claim 10 wherein the RBC/WBC ratio is in the range of about 0.2-250 and the RBC volume percent is in the range of about 1-20 in the WBC-rich fraction.

12. Method of Claim 11 wherein the RBC/WBC ratio is in the range of abut 0.2-50 and the RBC volume percent is in the range of about 1-6 in the WBC-rich fraction.

13. Method of Claim 12 wherein the differential of WBC-rich fraction is about 1-5% granulocytes, 0-20% monocytes and greater than about 80% lymphocytes.

14. Method of Claim 13 wherein the RBC/WBC ratio is in the range of about 0.5-25 and the RBC volume content is about 2-4 in the WBC-rich fraction.

15. In the method of treatment of a cancer patient by adoptive immunotherapy which comprises removing peripheral blood from the patient, separating a lymphocyte-containing WBC-rich fraction from the blood, incubating the lymphocyte-containing WBC-rich fraction with interleukin-2 to produce lymphokine-activated killer cells, and reinjecting the activated cells into the patient, the improvement which comprises separating the lymphocyte-containing WBC-rich fraction without use of a ficoll gradient, whereby the volume percent of RBC's in the WBC-rich fraction is in the range of about 1-20.

16. Method of Claim 15 wherein the lymphocyte-containing WBC-rich fraction is separated by elutriation leukapheresis whereby the volume percent of RBC's in the WBC fraction is in the range of about 1-6.

Revendications

1. Dans le procédé de production in vitro de lymphocytes-tueurs activés par lymphokine (LAK) consistant à extraire les globules rouges (RBC) et le plasma du sang entier afin de produire une fraction riche en globules blancs (WBC) contenant des lymphocytes et à incuber cette fraction riche en WBC dans un milieu de culture avec IL-2, l'amélioration qui consiste à extraire les RBC et le plasma et à utiliser la fraction riche en WBC sans séparation intermédiaire des lymphocytes sur un gradient de ficoll.

2. Procédé selon la revendication 1, dans lequel les RBC sont éliminés par leucophérèse et le pourcentage en volume des RBC dans la fraction riche en WBC est compris dans l'intervalle d'environ 1 à 20.

3. Procédé selon la revendication 2, dans lequel le rapport RBC/WBC dans la fraction riche en WBC est compris dans l'intervalle d'environ 0,2 à 250.

4. Procédé selon la revendication 1, dans lequel les RBC sont éliminés par leucophérèse par élutriation et le pourcentage en volume de RBC dans la fraction riche en WBC est compris dans l'intervalle d'environ 1 à 6.

5. Procédé selon la revendication 4, dans lequel le rapport RBC/WBC dans la fraction riche en WBC est compris dans l'intervalle d'environ 0,2 à 50.

6. Procédé selon la revendication 5, dans lequel la composition des WBC est d'environ 80 à 85% de lymphocytes, d'environ 10 à 20% de monocytes et d'environ 1 à 5% de granulocytes.

7. Procédé selon la revendication 6, dans lequel le pourcentage en volume des RBC dans la fraction riche en WBC est dans l'intervalle d'environ 2 à 4, le rapport RBC/WBC dans la fraction riche en WBC est

dans l'intervalle d'environ 0,5 à 25.

8. Procédé selon la revendication 1, dans lequel il y a appauvrissement en monocytes par traitement avec l'ester méthylique de la phénylalanine avant l'incubation de la fraction riche en WBC.

9. Procédé selon la revendication 2, dans lequel la fraction riche en WBC est lavée par une solution saline avant l'incubation afin d'inhiber la coagulation.

10. Dans le procédé de génération de cellules LAK par l'incubation d'une fraction riche en WBC contenant des lymphocytes dans un milieu de culture avec IL-2, l'amélioration qui consiste à utiliser une fraction, riche en WBC, contenant des lymphocytes ayant un rapport RBC/WBC, par nombre, dans l'intervallle d'environ 0,2 à 300 et un pourcentage en volume de RBC d'environ 1 à 50.

11. Procédé selon la revendication 10, dans lequel le rapport RBC/WBC est compris dans l'intervalle d'environ 0,2 à 250 et le pourcentage en volume des RBC est compris dans l'intervalle d'environ 1 à 20 dans la fraction riche en WBC.

12. Procédé selon la revendication 11, dans lequel le rapport RBC/WBC est compris dans l'intervalle d'environ 0,2 à 50 et le pourcentage en volume des RBC est compris dans l'intervalle d'environ 1 à 6 dans la fraction riche en WBC.

13. Procédé selon la revendication 12, dans lequel la composition de la fraction riche en WBC est d'environ 1 à 5% de granulocytes, 0 à 20% de monocytes et supérieure à environ 80% de lymphocytes.

14. Procédé selon la revendication 13, dans lequel le rapport RBC/WBC est compris dans l'intervalle d'environ 0,5 à 25 et la teneur en volume des RBC est d'environ 2 à 4 dans la fraction riche en WBC.

15. Dans le procédé de traitement d'un patient souffrant d'un cancer par immunothérapie adoptive consistant à enlever du sang circulant à la périphérie du patient, séparer une fraction riche en WBC contenant des lymphocytes à partir du sang, incuber la fraction riche en WBC contenant des lymphocytes avec interleucine-2 afin de produire des lymphocytestueurs activés par lymphokine, et réinjecter les cellules ainsi activées au patient, l'amélioration consiste à séparer la fraction, riche en WBC, contenant des lymphocytes, sans l'utilisation d'un gradient en ficoll de manière à ce que le pourcentage en volume des RBC dans la fraction riche en WBC est compris dans l'intervalle d'environ 1 à 20.

16. Le procédé selon la revendication 15, dans lequel la fraction riche en WBG contenant des lymphocytes est séparée par leucophérèse par élutriation de manière à ce que le pourcentage en volume des RBC dans la fraction WBC soit compris dans l'intervalle d'environ 1 à 6.

Patentansprüche

1. Verfahren zur Erzeugung von LAK-Zellen in vitro, umfassend das Entfernen der RBCs und des Plasmas aus Vollblut zur Gewinnung einer Lymphocyten enthaltenden, an WBCs reichen Fraktion und Inkubieren der WBC-reichen Fraktion in einem Kulturmedium mit IL-2, dadurch gekennzeichnet, daß die RBCs und das Plasma entfernt werden und die WBC-reiche Fraktion ohne intermediäre Abtrennung von Lymphocyten auf einem Ficoll-Gradienten verwendet wird.

2. Verfahren nach Anspruch 1, worin die RBCs durch Leukapherese entfernt werden und der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 20 Vol.-% liegt.

3. Verfahren nach Anspruch 2, worin das Verhältnis RBC/WBC in der WBC-reichen Fraktion im Bereich von etwa 0,2 bis 250 liegt.

4. Verfahren nach Anspruch 1, worin die RBCs durch Schlämm-Leukapherese entfernt werden und der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 6 Vol.-% liegt.

5. Verfahren nach Anspruch 4, worin das Verhältnis RBC/WBC in der WBC-reichen Fraktion im Bereich von etwa 0,2 bis 50 liegt.

6. Verfahren nach Anspruch 5, worin das WBC-Differential etwa 80 bis 85 % Lymphocyten, etwa 10 bis 20 % Monocyten und etwa 1 bis 5 % Granulocyten zeigt.

7. Verfahren nach Anspruch 6, worin der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 2 bis 4 Vol.-% liegt und das Verhältnis RBC/WBC in der WBC-reichen Fraktion im Bereich von etwa 0,5 bis 25 liegt.

8. Verfahren nach Anspruch 1, worin vor der Inkubation der WBC-reichen Fraktion die Monocyten durch Behandlung mit Phenylalaninmethylester abgereichert werden.

9. Verfahren nach Anspruch 2, worin die WBC-reiche Fraktion vor der Inkubation mit Salz-Lösung gewaschen wird, um eine Gerinnsel-Bildung zu hemmen.

10. Verfahren zur Erzeugung von LAK-Zellen durch Inkubieren einer Lymphocyten enthaltenden, WBC-reichen Fraktion in einem Kulturmedium mit IL-2, dadurch gekennzeichnet, daß eine Lymphocyten enthaltende, WBC-reiche Fraktion mit einem Zahlen-Verhältnis RBC/WBC im Bereich von etwa 0,2 bis 300 und einem Anteil der RBCs von etwa 1 bis 50 Vol.-% eingesetzt wird.

11. Verfahren nach Anspruch 10, worin das Verhältnis RBC/WBC im Bereich von etwa 0,2 bis 250 liegt und der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 20 Vol.-% liegt.

12. Verfahren nach Anspruch 11, worin das Verhältnis RBC/WBC im Bereich von etwa 0,2 bis 50 liegt und der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 6 Vol.-% liegt.

13. Verfahren nach Anspruch 12, worin das Differential der WBC-reichen Fraktion etwa 1 bis 5 % Granulocyten, 0 bis 20 % Monocyten und mehr als etwa 80 % Lymphocyten zeigt.

14. Verfahren nach Anspruch 13, worin das Verhältnis RBC/WBC im Bereich von etwa 0,5 bis 25 liegt und der Anteil der RBCs in der WBC-reichen Fraktion etwa 2 bis 4 Vol.-% beträgt.

15. Verfahren zur Behandlung eines Krebs-Patienten durch passive Immuntherapie, umfassend
die Entnahme von peripherem Blut des Patienten,
das Abtrennen einer Lymphocyten enthaltenden, an WBCs reichen Fraktion aus dem Blut,
das Inkubieren der Lymphocyten enthaltenden, WBC-reichen Fraktion mit Interleukin-2, um Lymphokin-aktivierte Killer-Zellen zu erzeugen, und
das Rückinjizieren der aktivierten Zellen in den Patienten,
dadurch gekennzeichnet, daß die Lymphocyten enthaltende, WBC-reiche Fraktion ohne Anwendung eines Ficoll-Gradienten abgetrennt wird, wodurch der Anteil der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 20 Vol.-% liegt.

16. Verfahren nach Anspruch 15, worin die Lymphocyten enthaltende, WBC-reiche Fraktion durch Schlämm-Leukapherese abgetrennt wird, wodurch der Anteil der RBCs in der WBC-reichen Fraktion im Bereich von etwa 1 bis 6 Vol.-% liegt.